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PEG Length and Chemical Linkage Controls Polyacridine Peptide DNA Polyplex Pharmacokinetics, Biodistribution, Metabolic Stability and *In Vivo* Gene Expression

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Abstract

The pharmacokinetics (PK), biodistribution and metabolism of non-viral gene delivery systems administered systemically are directly related to *in vivo* efficacy. The magnitude of luciferase expression in the liver of mice following a tail vein dose of a polyplex, composed of 1 μ g of pGL3 in complex with a polyethylene glycol (PEG) polyacridine peptide, followed by a delayed hydrodynamic (HD) stimulation (1–9 h), depends on the HD stimulation delay time and the structure of the polyacridine peptide. As demonstrated in the present study, the PEG length and the type of chemical linkage joining PEG to the polyacridine peptide dramatically influence the *in vivo* gene transfer efficiency. To understand how PEG length, linkage and location influence gene transfer efficiency, detailed PK, biodistribution and HD-stimulated gene expression experiments were performed on polyplexes prepared with an optimized polyacridine peptide modified through a single terminal Cys or Pen (penicillamine) with a PEG chain of average length of 2, 5, 10, 20, or 30 kDa. The chemical linkage was examined by attaching PEG_{5kDa} to the polyacridine peptide through a thiol-thiol (SS), thiol-maleimide (SM), thiol-vinylsulfone (SV), thiol-acetamide (SA), penicillamine-thiol-maleimide (PM) or penicillamine-thiol-thiol (PS). The influence of PEG location was analyzed by attaching PEG_{5kDa} to the polyacridine peptide through a C-terminal, N-terminal, or a middle Cys residue. The results established rapid metabolism of polyplexes containing SV and SA chemical linkages leads to a decreased polyplex PK half-life and a complete loss of HD-stimulated gene expression at delay times of 5 hrs. Conversely, polyplexes containing PM, PS, and SM chemical linkages were metabolically stable, allowing robust HD-stimulated expression at delay times up to 5 hrs post polyplex administration. The location of PEG_{5kDa} within the polyacridine peptide exerted only a minor influence on the gene transfer of polyplexes. However, varying the PEG length from 2, 5, 10, 20, or 30 kDa dramatically altered polyplex biodistribution, with a 30 kDa PEG maximally blocking liver uptake to 13% of dose, while maintaining the ability to mediate HD-stimulated gene expression. The combination of results establishes important relationships between PEGylated polyacridine peptide structure, physical properties, *in vivo* metabolism, PK and biodistribution resulting in an optimal PEG length and linkage that leads to robust HD-stimulated gene expression in mice.

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Keywords

Gene Delivery; Hydrodynamic Dosing; Polyethylene glycol

Introduction

The PEGylation of a nonviral gene delivery system is considered essential in order to improve blood compatibility of DNA polyplexes[1, 2]. Without PEGylation, upon i.v. administration the cationic surface of most polyplexes binds albumin and other serum proteins causing a rapid increase in particle size and entrapment in the capillary bed of the lung [3, 4]. In the absence of PEGylation, anionic polyplexes and plasmid DNA are rapidly removed from the circulation by liver Kupffer cells via the scavenger receptor [5, 6].

Several prior studies have examined the influence of PEG length and loading density on nanoparticle biodistribution *in vivo*[7–11]. In general, increasing the PEG length and loading density decreases serum protein binding and biodistribution to the lung. However, even with a stealth layer of PEG, polyplexes are still primarily recognized by Kupffer cells, accounting for the majority of biodistribution to liver immediately after i.v. administration[12]. Although these relationships are well understood, to date no studies have reported the development of long-circulating DNA nanoparticles that avoid liver uptake and maintain the ability to mediate gene transfer *in vivo*.

A major difficulty in PEGylating DNA polyplexes is that increasing the PEG loading density can also compromise polyplex stability. This is because PEGylation of PEI[13, 14], polylysine, chitosan[15] or dendrimers simultaneously results in a proportional decrease in the number of primary amines, thereby decreasing the binding affinity of PEGylated polymers for DNA, resulting in their dissociation in the circulation[16]. In an effort to overcome this limitation, PEGylated adamantane was incorporated through binding to cationic cyclodextrin polyplexes[17]. While this approach preserved amines to maintain binding affinity for DNA, it failed to achieve a high PEG loading density.

The cross-linking of residual amines on Lys residues of peptides with glutaraldehyde was previously used to stabilize PEGylated peptide DNA polyplexes from dissociation in the circulation[18]. Likewise, HPMA was used previously to cross-link amines on the surface of polyplexes resulting in laterally stabilized DNA nanoparticles that possessed a long circulatory half-life[19]. However, both of these approaches resulted in polyplexes that failed to mediate appreciable gene expression *in vivo*, presumably due in part to their inability to release DNA inside the cell [20, 21].

We have previously reported on the unique attributes of PEGylated polyacridine peptides that bind to DNA to form polyplexes that overcome the limitations of other strategies by providing a long circulatory half-life and demonstrated *in vivo* activity. In prior studies we first optimized the properties of the peptide portion of PEGylated polyacridine peptides to achieve efficient *in vivo* gene expression following a delayed hydrodynamic (HD)-stimulatory dose (saline only) after i.v. administration of the polyplex[22]. Short PEGylated polyacridine peptides of the general structure (Acr-Lys)_{n=2,4,6}-Cys, where Acr is a Lys residue modified on its ϵ -amine with acridine, were found to bind pGL3 with high affinity through a combination of polyintercalation and ionic interaction. PEGylation of the C-terminal Cys residue resulted in PEGylated polyacridine peptides that bind to pGL3 to form polyplexes that are stable in the circulation for up to two hrs[22]. Further optimization of the peptide sequence revealed that four Acr residues spaced by four Lys residues, (Acr-Lys₄)₃-Acr-Lys-Cys-PEG, dramatically increased the stability of a 1 μ g i.v. dose of pGL3 polyplex in the circulation for 5 hrs[23].

The present investigation examines the influence of PEG length, linkage and location on DNA polyplex pharmacokinetics (PK), biodistribution and gene expression *in vivo*. The identified relationships were found using high-affinity DNA binding polyacridine peptides possessing a single Cys or Pen attachment site for PEG [22, 23]. A PEG was linked to either Cys or Pen using six different chemical linkages which included thiol-maleimide (**SM**), thiol-thiol (**SS**), thiol-acetamide (**SA**), thiol-vinyl sulfone (**SV**), penicillamine-thiol-thiol (**PS**), and penicillamine-thiol-maleimide (**PM**). In addition, five different PEG lengths (2, 5, 10, 20, and 30 kDa) and three different attachment sites (N-terminal, C-terminal and middle) were compared for their influence on polyplex mediated *in vivo* gene delivery.

The results are startling in that linkages anticipated to be most stable (**SA**, **SV**) were found to result in short PK half-lives and rapid loss of HD-stimulated gene expression. Likewise, linkages expected to be metabolically less stable (**SS**, **PS**) proved to be more stable than **SA** and **SV**, resulting in longer PK half-lives and extended HD-stimulated expression profiles. Just as important as PEG linkage, PEG length was found to exert a dramatic effect on PK half-life and the ability to stealth polyplex biodistribution to the liver, resulting in the discovery of DNA polyplexes with a long circulatory half-life that avoid biodistribution to the liver. Together, the results address key parameters regarding the attachment of PEG to DNA polyplexes that improve the performance of i.v. dosed non-viral gene delivery systems in mice.

Materials and Methods

Unsubstituted Wang resin, 9-hydroxybenzotriazole, Fmoc-protected amino acids, O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU), Fmoc-Lysine-OH, and N-Methyl-2-pyrrolidone (NMP) were obtained from AAPPTec (Louisville, KY, USA). Fmoc Penicillamine(trityl)-Wang (Fmoc-Pen) was from BACHEM, (King of Prussia, PA). N, N-Dimethylformamide (DMF), trifluoroacetic acid (TFA), and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Diisopropylethylamine, piperidine, acetic anhydride, Tris (2-carboxyethyl)-phosphine hydrochloride (TCEP), 9-chloroacridine, thiazole orange, N-hydroxysuccinimide (NHS), iodoacetic acid and dicyclohexylcarbodiimide (DCC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Agarose was obtained from Gibco-BRL (Carlsbad, CA, USA). mPEG-maleimide (2, 5, 10, 20, 30 kDa), mPEG_{5kDa}-S-2-sulfanylpuridine and mPEG_{5kDa}-amine were purchased from Laysan Bio (Arab, AL, USA), whereas mPEG_{5kDa}-vinylsulfone was from Jenkem Technologies, Beijing, China. D-Luciferin and luciferase from *Photinus pyralis* were obtained from Roche Applied Science (Indianapolis, IN, USA). pGL3 control vector, a 5.3 kbp luciferase plasmid containing a SV40 promoter and enhancer, was obtained from Promega (Madison, WI, USA). pGL3 was amplified in a DH5 α strain of *Escherichia coli* and purified according to the manufacturer's instructions.

Synthesis and Characterization of PEGylated Polyacridine Peptides

9-Phenoxyacridine and Fmoc-Lysine(Acridine)-OH were prepared as recently reported [24, 25]. The polyacridine peptides defined in Figure 1 and Table 1S were prepared by solid phase peptide synthesis on a 30 μ mol scale using an APEX 396 synthesizer (Advanced ChemTech, Louisville, KY, USA) with standard Fmoc procedures.

PEGylation of the Cys residue on (Acr-Lys₄)₃-Acr-Lys-Cys was achieved by reacting 1 μ mol of peptide with 1.1 μ mol of PEG_{5kDa}-maleimide in 4 ml of 100 mM HEPES buffer pH 7 for 12 hrs to generate (Acr-Lys₄)₃-Acr-Lys-Cys-Mal-PEG_{5kDa} (**SM1_{5kDa}**). In addition, S-Mal PEG-peptides were prepared with PEG_{2kDa} (**SM1_{2kDa}**), PEG_{10kDa} (**SM1_{10kDa}**), PEG_{20kDa} (**SM1_{20kDa}**) and PEG_{30kDa} (**SM1_{30kDa}**) (Table 1S).

Similarly, 1 μmol of $(\text{Acr-Lys}_4)_3\text{-Acr-Lys-Pen}$ was reacted with 1.1 μmol of $\text{PEG}_{5\text{kDa}}\text{-maleimide}$ to form $(\text{Acr-Lys}_4)_3\text{-Acr-Lys-Pen-Mal-PEG}_{5\text{kDa}}$ (**PM**). Reaction of 1 μmol of $(\text{Acr-Lys}_4)_3\text{-Acr-Lys-Cys/Pen}$ with 1.1 μmol of $\text{PEG}_{5\text{kDa}}\text{-S-2-sulfanyl pyridine}$ in 4 ml of 100 mM HEPES pH 7 for 12 hrs resulted in the formation of $(\text{Acr-Lys}_4)_3\text{-Acr-Lys-Cys-SS-PEG}_{5\text{kDa}}$ (**SS**), and $(\text{Acr-Lys}_4)_3\text{-Acr-Lys-Pen-SS-PEG}_{5\text{kDa}}$ (**PS**). The optimized reaction to generate $(\text{Acr-Lys}_4)_3\text{-Acr-Lys-Cys-SV-PEG}_{5\text{kDa}}$ (**SV**) was performed by adding 1 μmol of $(\text{Acr-Lys}_4)_3\text{-Acr-Lys-Cys}$ and 10 μmol of $\text{PEG}_{5\text{kDa}}\text{-vinyl sulfone}$ in 4 ml of 100 mM HEPES pH 7.5, which was allowed to react for 12 hrs.

The formation of a PEG-peptide with an iodoacetamide linkage was achieved by derivatization of amino- $\text{PEG}_{5\text{kDa}}$ with NHS-iodoacetic acid. Amino- $\text{PEG}_{5\text{kDa}}$ (5 μmol s) was reacted with 50 μmol s of NHS-iodoacetic acid in 5 ml of 0.1 M sodium bicarbonate pH 8.0 for 5 hrs. The reaction product was isolated by Sephadex G10 column chromatography (2.5 $\times$ 50 cm) eluted with 0.1 v/v % TFA by collecting the peak eluting at 100 ml, which was concentrated by rotary evaporation, freeze dried, reconstituted in water and quantified by $\text{Abs}_{280\text{nm}}$ of iodoacetamide ($\epsilon = 370 \text{ M}^{-1}\text{cm}^{-1}$). Analytical reverse phase high performance liquid chromatographic (RP-HPLC) analysis of the product (10 nmol)s eluted at 1 ml min^{-1} with 0.1 v/v % TFA with a gradient of acetonitrile of 15–65 v/v % over 30 min while monitoring $\text{Abs}_{280\text{nm}}$ identified iodoacetamide-PEG eluting at 19 min. Reaction of 1 μmol of $(\text{Acr-Lys}_4)_3\text{-Acr-Lys-Cys}$ with 5 μmol s of iodoacetamide-PEG in a total volume of 4 ml of 0.1 M sodium bicarbonate resulted in the conversion of the peptide into $(\text{Acr-Lys}_4)_3\text{-Acr-Lys-Cys-S-Acetamide-PEG}$ (**SA**). PEGylated peptides were purified by semi-preparative RP-HPLC as eluted at 10 ml min^{-1} with 0.1 v/v % TFA and a gradient of 20–60 v/v % acetonitrile while monitoring acridine absorbance at $\text{Abs}_{409\text{nm}}$. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized, and stored at -20°C . The counter ion was exchanged by chromatography on a Sephadex G-25 column (2.5 $\times$ 50 cm) equilibrated with 0.1 v/v % acetic acid to obtain the peptide in an acetate salt form. The major peak corresponding to the PEG-peptide eluting at 100 ml was pooled, concentrated by rotary evaporation, and freeze-dried. PEG-peptides were reconstituted in water and quantified by $\text{Abs}_{409\text{nm}}$ to determine isolated yield (Table 1S). PEG-peptides were characterized by matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF MS) by combining 1 nmol with 10 μl of 2 mg ml^{-1} α -cyano-4-hydroxycinnamic acid (CHCA) in 50 v/v % acetonitrile and 0.1 v/v % TFA. Samples were spotted onto the target and ionized on a Bruker Biflex III Mass Spectrometer (Bruker Daltonics Inc, Billerica, MA, USA) operated in the positive ion mode.

Formulation and In Vivo Analysis of PEGylated Polyacridine Peptide Polyplexes

The relative binding affinity of PEGylated polyacridine peptides for pGL3 was determined by a fluorophore exclusion assay[26]. Pharmacokinetic and biodistribution analysis of PEGylated polyacridine polyplexes were performed using radioiodinated pGL3 as previously described [22, 23, 27]. HD-stimulated expression of luciferase with quantitative bioluminescence imaging in liver were performed as previously described [22, 23].

The metabolic stability of PEGylated polyacridine peptides was determined by incubating 10 nmol with 500 μl of either EDTA (2 mg ml^{-1}) whole mouse blood, freshly prepared mouse liver or spleen homogenate prepared in 0.25 M sucrose[28]. After incubation at 37°C for 4 h, the blood, liver or spleen homogenates were precipitated by adding 250 μl of lysate with 125 μl 20 v/v% TFA/ ddH_2O , followed by incubation at 4°C for 30 min, and then centrifuging at 10,000 g for 30 min. The soluble fraction containing the PEGylated acridine peptide was analyzed by $\text{Abs}_{409\text{nm}}$ to determine recovery (95%). Recovered PEGylated peptides were analyzed by RP-HPLC eluted at 1 ml min^{-1} with 0.1% TFA and a gradient of 15–60% acetonitrile over 30 min while detecting at $\text{Abs}_{409\text{nm}}$. The retention time of PEG-

peptide digests were compared to untreated PEG-peptide standards and a non-PEGylated peptide to determine metabolic status.

Results

The development of nonviral gene delivery systems that function *in vivo* requires careful optimization using realistic doses in animal models with reliable gene expression readouts. We have chosen to optimize PEGylated polyacridine peptide delivery systems using a standardized tail vein dose of 1 μg of pGL3 in 50 μl in 15–20 g male ICR mice. pGL3 is a standard commercially available 5.3 kbp plasmid expressing luciferase under the control of an SV40 promoter.

It has been previously demonstrated that pGL3 is rapidly metabolized by endogenous DNase in the blood resulting in no detectable luciferase expression when delivered i.v. to mice [18, 22, 23]. Alternatively, a rapid (5 sec) direct-hydrodynamic dose of 1 μg of pGL3 in approximately 1.8 ml of saline via the tail vein in a 20 g mouse results in the bioluminescence imaging (BLI) detection of $10^8 \text{ photons sec}^{-1} \text{ cm}^{-2} \text{ seradian}^{-1}$ for luciferase in the liver after 24 h [22, 29]. Results were analyzed for statistical significance ($p < 0.05$) based on Dunnett T3 test using SPSS 19.0 analysis of variance software.

The delivery of 1 μg of pGL3 PEGylated polyacridine polyplex to mice via the tail vein in 50 μl also leads to no detectable expression in the liver. However, following polyplex delivery, the administration of a blank (saline only) HD-stimulatory dose at times ranging from 5 min to 9 hrs post-DNA delivery, results in appreciable luciferase expression in the liver. Both the magnitude of expression and the length of the stimulation time window are influenced by the structure of the PEGylated polyacridine peptide [22, 23]. Increasing the affinity of PEGylated polyacridine peptides for DNA results in polyplexes with greater stability in the circulation and a longer stimulation time window [22, 23]. Using an optimized PEGylated polyacridine peptide polyplex, the magnitude of expression mediated by HD-stimulation is equivalent to that of direct-HD dosing of 1 μg of pGL3 [22].

In the present study we examined the influence of PEG linkage by attaching PEG_{5kDa} to (Acr-Lys₄)₃-Acr-Lys-Cys/Pen (**1** or **2**, Table 1S), using six unique linkages which included thiol-maleimide (**SM**), thiol-thiol (**SS**), thiol-acetamide (**SA**), thiol-vinyl sulfone (**SV**), penicillamine-thiol-maleimide (**PM**), and penicillamine-thiol-thiol (**PS**) (Fig. 1, Table 1S). PEGylated polyacridine peptides were prepared by first synthesizing peptides **1–4**, which were RP-HPLC purified in approximately 20% isolated yield and characterized by LC-ESI-MS prior to derivatization with PEG (Table 1S).

Monofunctionalized PEGs with a terminal maleimide, S-2-sulfanylpuridine, vinyl sulfone, or amine were selected based on their commercial availability. A maleimide-PEG_{5kDa} was reacted with the C-terminal Cys or Pen on **1** or **2** to produce PEG-peptides **SM1_{5kDa}** and **PM** (Fig. 1A). An S-2-sulfanylpuridine-PEG_{5kDa} was reacted with a C-terminal Cys or Pen to produce PEG-peptides **SS** and **PS** (Fig. 1A). A vinyl sulfone PEG_{5kDa} was reacted with the C-terminal of Cys to produce **SV**. Conversion of an amino-PEG_{5kDa} to an iodoacetamide, followed by reaction with a C-terminal Cys produced **SA** (Fig. 1A). In addition, a control non-PEGylated polyacridine peptide was prepared by alkylating the Cys on peptide **1** to form **S_{alk}** (Fig. 1A, Table 1S). Furthermore, the influence of PEG length on polyplex PK and *in vivo* gene transfer was evaluated by modification of peptide **1** with maleimide modified PEG_{2, 5, 10, 20, or 30kDa} to prepare **SM1_{2kDa}**, **SM1_{5kDa}**, **SM1_{10kDa}**, **SM1_{20kDa}** and **SM1_{30kDa}** (Fig. 1A). In addition, peptides **3** and **4** were designed as geometric isomers of peptide **1** with either an N-terminal or middle Cys residue. Peptides **3** and **4** were reacted with maleimide-PEG_{5kDa} to prepare **SM3_{5kDa}** and **SM4_{5kDa}**, to test the

influence of PEG location on the ability of PEGylated polyacridine peptides to mediate gene expression *in vivo* (Fig. 1B and C and Table 1S).

PEG-peptides **SM1**_{2kDa}, **SM1**_{5kDa}, **SM1**_{10kDa}, **SM1**_{20kDa}, **SM1**_{30kDa}, **SM3**_{5kDa}, **SM4**_{5kDa}, **PM**, **SS**, **PS**, **SV**, **SA** and **S_{alk}** were isolated using preparative RP-HPLC in yields ranging from 26–86 % based on peptides **1–4**. MALDI-TOF MS analysis of PEGylated polyacridine peptides resulted in observed masses ranging from 5174–36459 Da. The difference between the calculated and observed mass is related to PEG polydispersity in the commercially available functionalized PEGs. The ability of PEGylated polyacridine peptides to bind pGL3 was evaluated using a thiazole orange dye exclusion assay [26]. The results established that each PEG-peptide completely displaced the fluorophore at a stoichiometry of 0.15 nmol μg^{-1} of pGL3, suggesting that the identity of the linker joining PEG to the peptide did not influence DNA binding affinity (Fig. 2A). However, the location of PEG exerted a minor influence on the ability of PEG-peptides to displace thiazole orange (Fig. 2A).

Attachment of PEG to Cys located in the middle (**SM4**_{5kDa}) or N-terminus (**SM3**_{5kDa}) of the polyacridine peptide resulted in slightly lower apparent affinities for pGL3 based on the decreased ability of these peptides to displace thiazole orange from DNA at peptide stoichiometries of 0.05–0.1 nmol μg^{-1} of pGL3 (Fig. 2A). Similarly, a PEG length of 2, 20, or 30 kDa resulted in a similar decrease in affinity at low stoichiometries for **SM1**_{2kDa}, **SM1**_{20kDa} and **SM1**_{30kDa} relative to **SM1**_{5kDa} and **SM1**_{10kDa} (Fig. 2B).

We have previously shown that PEGylated polyacridine peptides form DNase stable electronegative polyplexes at a stoichiometry of 0.2 nmol μg^{-1} of pGL3, but that further addition of PEG-peptide results in more fully condensed electropositive DNase stable polyplexes at 0.8 nmols of peptide μg^{-1} of pGL3[22]. While both electronegative and electropositive polyplexes are able to mediate gene expression *in vivo* following hydrodynamic stimulation, electropositive polyplexes are more tightly condensed and protect pGL3 from DNase in the circulation for a longer time [23].

For this reason, the relative particle size and zeta potential were compared for polyplexes prepared at a standard stoichiometry 0.8 nmols of peptide μg^{-1} of pGL3 (Fig. 3). This stoichiometry was also used for all PK, biodistribution and *in vivo* expression experiments. Polyplexes prepared with **PM**, **SM**, **PS**, **SS**, **SV** and **SA** possessed a mean diameter of 170–200 nm (Fig. 3A) whereas polyplexes prepared with **S_{alk}** were determined to be smaller, with a diameter of 105 nm (Fig. 3B). Alternatively, polyplexes prepared with **SM1**_{2kDa}, **SM1**_{5kDa}, and **SM1**_{10kDa} were approximately 170 nm in diameter, whereas **SM1**_{20kDa} and **SM1**_{30kDa} produced polyplexes that were 200 and 250 nm in diameter, respectively (Fig. 3B). The zeta potential of polyplexes prepared with **PM**, **SM**, **PS**, **SS**, **SV** and **SA** ranged from +10–14 mV and was independent of PEG-linkage and PEG-location (Fig. 3C). In contrast, polyplexes prepared with either **S_{alk}** or **SM1**_{2kDa} both produced zeta potentials of +28–30 mV, establishing that relative to longer PEGs, a 2kDa PEG was unable to mask polyplex charge (Fig. 3D). A significantly lower zeta potential of +10 mV was achieved using 5 and 10 kDa PEG on **SM1**_{5kDa} and **SM1**_{10kDa} (Fig. 3D). Increasing the PEG length further to 20 or 30kDa using **SM1**_{20kDa} and **SM1**_{30kDa} to form polyplexes resulted in progressive decrease in zeta potential to +5 and 0 mV, respectively (Fig. 3D).

The ability of **PM**, **SM**, **PS**, **SS**, **SV**, and **SA** polyplexes to mediate stimulated gene expression as a function of circulation time were compared (Fig. 4). The results revealed that **PM**, **SM** and **PS** each produced maximal gene expression of 10^8 photons $\text{sec}^{-1}\text{cm}^{-2}\text{steradian}^{-1}$ when applying HD-stimulation at times up to 4–5 h post DNA polyplex delivery (Fig. 4A).

The stimulated expression profiles from time zero to 4–5 h were stable suggesting the polyplexes were stable in the circulation, followed by a steady-decay in expression to background when applying HD-stimulation at times ranging from 5–9 hrs. The loss of gene expression at stimulation times greater than 5 hrs is most likely the result of peptide shedding and exposing polyplexes to DNase in the circulation as described previously[23]. The **SS** PEG-peptide polyplex demonstrated maximal expression of 10^8 photons $\text{sec}^{-1}\text{cm}^{-2}\text{ser}^{-1}$ at stimulation times up to 2 hrs, followed by a decay to zero at stimulation times of 2–5 hrs (Fig 4A). Surprisingly, **SA** and **SV** each produced ten-fold lower expression at 1 h relative to **PM**, **SM**, **PS** and **SS**, suggesting a decreased stability. The expression profile for **SA** revealed maximal expression of 10^7 photons $\text{sec}^{-1}\text{cm}^{-2}\text{seradian}^{-1}$ when applying HD-stimulation at times ranging from 1 to 3 hrs post-polyplex delivery, followed by a decay to background at 3–5 hrs (Fig. 4A). By comparison, **SV** PEG-peptide polyplexes only achieved 10^7 photons $\text{sec}^{-1}\text{cm}^{-2}\text{seradian}^{-1}$ after hydrodynamic stimulation times of 1 hr followed by a decay in expression to background at stimulation times of 2 hrs (Fig. 4A).

The unexpected rapid loss of HD-stimulated gene expression for **SV** and **SA** PEG-peptide polyplexes was in part explained by the rapid loss of pGL3 polyplexes from the blood. PK analysis revealed a much shorter α half-life for **SA** and **SV** polyplexes relative to **PM**, **PS** and **SS** (Fig. 5A, Table 2S). The short α half-life is similar to that determined for naked pGL3 which is rapidly acted upon by DNase in the circulation (Fig. 5A)[22, 23]. In contrast, the longer α half-life of **PM**, **PS** and **SS** polyplexes correlated with their greater stability in the circulation and their extended stimulated expression (Fig. 4A, 5A). Gel electrophoretic and autoradiographic analysis of blood time points confirmed that **SV** and **SA** polyplexes were also rapidly degraded (Fig. 5). An attempt to determine if polyplex metabolism occurred in the blood, liver or spleen resulted in no detectable metabolism of PEGylated peptides **SV** and **SA** when analyzing blood or tissue homogenate digests of polyplexes in vitro by RP-HPLC. The only metabolically sensitive PEG-peptides were **SS** and **PS** which underwent partial reduction of the disulfide in liver and spleen homogenate, presumably due to reduction by glutathione.

Comparison of **SM1_{10kDa}**, **SM1_{20kDa}** and **SM1_{30kDa}** polyplexes established that each mediated a 5-fold lower HD-stimulated gene expression relative to **SM1_{5kDa}** polyplexes at a delay time of 1hr (Fig. 4B). Delay times of up to 4 hrs post-polyplex delivery resulted in a stable level of expression for **SM1_{5kDa}** polyplexes, which then declined to background over 5 h. Alternatively, **SM1_{2kDa}** polyplexes mediated nearly 500-fold lower gene expression compared to **SM1_{5kDa}** polyplexes. The HD-stimulated expression profile was stable for 3 hrs and then rapidly declined to background (Fig. 4B).

The differences in the magnitude of HD-stimulated gene expression when varying PEG length were partially explained by analysis of the polyplex PK and biodistribution. **SM1_{2kDa}** polyplexes possessed the shortest α half-life, whereas **SM1_{5kDa}**, **SM1_{10kDa}**, **SM1_{20kDa}** and **SM1_{30kDa}** polyplexes possessed a coincident PK profile (Fig 5B, Table 2S).

The short α half-life of **SM1_{2kDa}** polyplexes is likely the result of the higher (+30 mV) surface charge revealed by zeta potential analysis (Fig. 3D) which correlates with greater liver uptake (70% of dose) compared to 50% of dose for **SM1_{5kDa}** (Fig. 6B').

Presumably, this is due to Kupffer cell recognition of the electropositive polyplex. Interestingly, as the PEG length was increased from 10 to 30 kDa, the percent of dose captured by the liver decreased, resulting in only 13% of dose associated with liver following administration of **SM1_{30kDa}** polyplexes (Fig. 6B'). The biodistribution of **SM1_{20kDa}** polyplexes resulted in an increase in spleen uptake, which reached a maximum of 18% of dose at a biodistribution time of 2 hr (Fig. 6D, Table 3S).

The fundamental importance of PEG to protect polyplexes from protein binding, Kupffer cell recognition in the liver, uptake in the spleen, or entrapment in the lung *in vivo* can be ascertained by analysis of the PK, biodistribution and stimulated expression for **S_{alk}** polyplexes. The complete loss of stimulated gene expression (Fig. 4C) is directly correlated with rapid and nearly complete loss of **S_{alk}** polyplexes from the blood as revealed by PK analysis (Fig. 5C). The biodistribution properties of **S_{alk}** polyplexes are distinctive in their immediate uptake by the liver and lung (Fig. 6B and C), with a high percentage of the dose (30%) in lung that remains constant over time (Fig. 6C). This biodistribution profile is characteristic of a stable cationic non-PEGylated polyplex that ionically attracts albumin, resulting in larger particles that become entrapped in the capillary beds of the lung[30–32].

Attaching PEG via a maleimide linkage to either the C-terminal (**SM1_{5kDa}**) or N-terminal (**SM3_{5kDa}**) Cys residue resulted in only small differences in DNA binding affinity (Fig. 2A), and no significant change in particle size, zeta potential (Fig. 3A and C), PKs (Fig. 5C) or HD-stimulated gene expression profiles (Fig. 4C). While attachment of PEG to a Cys placed in the center of the polyacridine peptide (**SM4_{5kDa}**) resulted in a slight decrease in the apparent DNA binding affinity (Fig. 2A), this did not influence the particle size, zeta potential (Fig. 3A and C) or PKs (Fig. 5C). **SM4_{5kDa}** polyplexes did mediate 5-fold less expression versus **SM1_{5kDa}** and **SM3_{5kDa}** polyplexes at HD-stimulation times of 4, 5, and 7 hrs (Fig. 4C). Thereby, it can be concluded that PEG attachment at the C or N-terminus has a negligible effect on polyplex performance in HD-stimulated gene expression whereas placement of the PEG in the middle of polyacridine peptide results in decreased gene transfer efficiency.

Discussion

Optimization of the PK and biodistribution properties of non-viral gene delivery systems administered systemically is key to their successful improvement in mediating gene transfer *in vivo*. Until now, there have been few, if any, delivery platforms that allowed systematic manipulation of PK and biodistribution properties to improve the level of gene expression *in vivo*. The PEGylated polyacridine peptide polyplex delivery platform with delayed HD-stimulation is unique in its ability to match the efficiency of direct-HD dosing of plasmid DNA [29, 33]. The application of HD-stimulation to complete the delivery of DNA to the liver at extended times is a highly stringent experimental tool to establish the viability of a gene delivery system during circulation. Consequently, it has been established that many commonly used gene delivery systems such as PEI, Lipofectamine, chitosan and PEGylated polylysine peptides fail to produce appreciable gene expression following HD-stimulation, even at very short circulation times [23]. While HD-stimulation has utility in facilitating the optimization of the stability and circulatory properties of gene formulations in mice, it is essential to develop more medically acceptable methods that replace HD-stimulation to complete gene delivery in order to find utility in humans.

In a prior study we systematically improved the polyacridine peptide design by increasing the spacing of four Acr residues with four Lys residues to arrive at (Acr-Lys₄)₃-Acr-Lys-Cys-PEG_{5kDa} (**SM1_{5kDa}**)[23]. pGL3 polyplexes prepared with **SM1_{5kDa}** mediated maximal expression when applying HD-stimulation at up to 4 hrs post-polyplex administration[23]. In the present study we have examined the influence of PEG length, linkage and location on polyacridine peptide mediated gene delivery. Notably, polyplexes prepared with **S_{alk}**, which lacks PEG, were completely inactive in mediating *in vivo* gene expression (Fig. 4C). The rapid removal of **S_{alk}** polyplexes from the blood is likely the result of serum protein binding to these polyplexes with a +30 mV surface charge, resulting in very rapid and complete loss from the blood, and combined accumulation of 83% of the dose in the liver and lung. It is clear that even though a large percentage (50%) of the dose is associated with liver for up to

60 min following systemic delivery, HD-stimulation fails to mediate any luciferase expression, suggesting that entrapment of cationic polyplexes by liver Kupffer cells inhibits HD-stimulated gene expression.

The PEG length is known to influence the PK of liposomal formulations[34]; however this relationship is less well defined for DNA polyplexes. The results establish that **SM1_{2kDa}** mediates a significantly shortened PK half-life, increased liver uptake (70%) and 100-fold loss in HD-stimulated gene expression relative to **SM1_{5kDa}**. The reason why **SM1_{10, 20 and 30 kDa}** mediate 5-fold lower gene expression relative to **SM1_{5kDa}** at delay times of 1 hr is not clear, but is most likely due to less efficient delivery to hepatocytes and release of pGL3 in the nucleus, and not related to biodistribution or premature shedding of PEG-peptide in the circulation. The finding that a 30 kDa PEG is able to stealth DNA polyplexes to nearly completely avoid liver uptake (13 % of dose) is of fundamental importance for the development of targeted delivery systems[35]. A clear systematic progression of decreasing liver uptake as PEG length increases suggests that stable polyplexes with a stealth layer of 30 kDa PEG can avoid Kupffer cell recognition in the liver (Fig. 6B').

The influence of PEG location on a peptide in relationship to polyplex PK and *in vivo* gene expression has not been previously reported. Locating PEG_{5kDa} on the C-terminal, N-terminal residue on a polyacridine peptide results in no detectable change in PK or level of HD-stimulated gene expression (Fig. 4C, 5C). Conversely locating the PEG in the middle of the peptide does not influence the level of gene expression at HD-stimulation times of 1 hr but does decrease expression five-fold at HD-stimulation times of 4–7 hours. The reason for the observed decrease in expression is likely due to a weaker binding affinity of the peptide for DNA, caused by a steric interruption of the binding sequence of four acridines spaced by four Lys residues with PEG.

With the exception of linkers designed to undergo reductive or pH sensitive cleavage [36, 37], few studies have considered the ramification of linker metabolic stability when attaching PEG to peptides or polymers used for *in vivo* gene delivery. It is stunning to realize that such subtle changes can have a dramatic influence on the efficiency of *in vivo* gene transfer. We presumed that the PEG linker is sterically inaccessible to metabolic enzymes and would have little influence over gene transfer efficiency. It is most likely that the mechanism involves dissociation of the PEG-peptide, followed by metabolism to release PEG from the peptide. Deactivation could result from DNase digestion of partially uncondensed polyplex. For **SV** and **SA** the metabolic deactivation is surprisingly fast, especially considering that a thiol-vinyl sulfone and thiol-acetamide linkage are significantly more chemically stable to reduction and elimination compared to **SS**, **PS**, **PM** and **SM**. However, the PK and HD-stimulation results establish **SV** and **SA** polyplexes have both a short half-life in the circulation and are much less active in mediating gene expression. We can deduce that **SV** and **SA** PEG-peptide metabolism is not occurring in the blood, since the de-shielded polyplex products would most likely accumulate in the lung, as was observed for **S_{alk}**. We hypothesize that **SV** and **SA** polyplexes are metabolized in liver Kupffer cells, where both removal of PEG and digestion of pGL3 by DNase could occur. This hypothesis is supported by biodistribution results that establish that **SV** and **SA** polyplexes highly accumulate in the liver (66%) and are eliminated at a similar fast rate compared to other polyplexes.

The comparative metabolic stability of **SS** polyplexes established that disulfide bond reduction occurs more slowly than metabolism of **SV** and **SA**. However, it also leads to a shortened PK half-life and HD-stimulated expression profile. The magnitude of gene expression mediated by **SS** polyplexes at 1 and 2 hr HD-stimulation time was nearly

coincident with that determined for **SM**, indicating PEG is likely not removed by intracellular reduction under the rapid delivery mediated by HD-stimulation. However, the loss of hydrodynamic stimulated expression for **SS** polyplexes at circulation times of longer than 2 hrs may be overcome by incorporation of a more reductively stable **PS** bond [38]. The results clearly establish that both **SM** and **PM** are the most metabolically stable chemical linkages resulting in long PK half-lives and an extended HD-stimulation profile.

It is likely that the underlying mechanism of HD-stimulated gene expression is the same as direct-HD dosing [39–42], both of which transfect liver hepatocytes following a rapid delivery of a large volume of saline. Likewise, it is also likely that direct-HD and HD-stimulation both by-pass all of the intracellular barriers to gene delivery. The primary difference is that HD-stimulation utilizes PEGylated polyplexes that are stable in the circulation for hrs, whereas direct-HD dosing uses naked plasmid DNA which has a metabolic half-life of minutes in the circulation.

In conclusion, an optimal PEG length of 5 kDa linked to a C or N terminal Cys through a maleimide linkage (**SM1_{5kDa}**), results in PK and biodistribution properties that produce maximal HD-stimulation when applied at up to 4–5 h following post-DNA delivery. However, even though increasing the PEG length to 30 kDa blocks most of the liver uptake, the majority of the stimulated gene expression in the liver is retained. The reported relationships between PK, biodistribution, metabolism and gene expression should be generally applicable for optimizing other PEGylated DNA and RNAi delivery systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structure of PEGylated Polyacridine Peptides

The structure of PEGylated polyacridine peptides used for gene delivery are illustrated. Peptide 1 (Table 1S) was modified with PEG of varying length to generate (Cys-Maleimide) SM1_{2kDa}, SM1_{5kDa}, SM1_{10kDa}, SM1_{20kDa} and SM1_{30kDa}. Peptide 1 or 2 were reacted with PEG_{5kDa} to generate SS (thiol-thiol), PS (penicillamine-thiol-thiol), PM (penicillamine-thiol-maleimide), SV (thiol-vinyl sulfone) and SA (thiol-acetamide). In addition, S_{alk} was generated by reacting the Cys on peptide 1 with iodoacetic acid. Peptide 3 and 4 (Table 1S) were reacted with maleimide-PEG_{5kDa} to generate geometric isomers of SM1_{5kDa} possessing PEG at the N-terminus (SM3_{5kDa}) and in the middle (SM4_{5kDa}) of the polyacridine peptide.

Figure 2. Binding of PEGylated Polyacridine Peptides to DNA

The displacement of thiazole orange from pGL3 was monitored by loss of fluorescence intensity as a function of peptide to DNA stoichiometry. Panel A illustrates the results of titrating SS, PS, PM, SV, S_{alk}, SM1_{5kDa}, SM3_{5kDa} and SM4_{5kDa}. Panel B illustrates the result of titrating SM1_{2kDa}, SM1_{5kDa}, SM1_{10kDa} and SM1_{20kDa}, and SM1_{30kDa}. Each result represents the mean and standard deviation of three determinations.

Figure 3. Size and Charge of PEGylated Polyacridine Peptide Polyplexes

The mean particle size and charge for pGL3 polyplexes ($30 \mu\text{g ml}^{-1}$) prepared at 0.8 nmol of PEG-peptide per μg of pGL3 determined by QELS are illustrated. Panels A and B establish the mean particle size of $105\text{--}250 \text{ nm}$ for varying PEG-peptide linkage, location of PEG, and PEG length. Panel C illustrates a mean zeta potential peptide polyplex possessing a 5 kDa PEG with different linkages. Panel D illustrates the zeta potential of peptide polyplexes with a maleimide linkage with different PEG length. Each result represents the mean and standard deviation of three determinations.

Figure 4. HD-Stimulated Gene Expression

The relative gene expression in the liver was determined after tail vein administration of a 1 μ g dose of pGL3 peptide polyplex in triplicate mice followed by delayed HD-stimulation (tail vein administration of 1.5–2 ml of saline in 5 sec) at times ranging from 1–9 hrs. The magnitude of luciferase expression was determined by BLI at 24 hrs after HD-stimulation. Panel A illustrates the influence of varying PEG_{5kDa}-peptide linkage (SM, PM, PS, PS, SS, SA, SV) to peptide 1. Panel B illustrates the influence of varying PEG length (2, 5, 10, 20, and 30 kDa) linked to peptide 1 with a maleimide linkage. Panel C illustrates the influence of C (SM1) versus N-terminal (SM3), versus middle (SM4) attachment of PEG_{5kDa} to peptide 1 via a maleimide linkage, relative to no PEG (Salk). Each data point represents the mean and standard deviation resulting from BLI measured for three independently dosed mice. At 1 hr the asterisks indicate a statistical significance of $p < 0.05$ compared with SM1_{5kDa} (+).

Figure 5. Pharmacokinetic Analysis of PEGylated Polyacridine Peptide Polyplexes
 The concentration of ^{125}I -pGL3 polyplexes and ^{125}I -pGL3 in the circulation versus time is compared as a function of differing PEG-peptide. Panel A illustrates the influence of PEG-peptide linkage by comparing the PK profile for **PM**, **PS**, **SS**, **SV**, and **SA** polyplexes, relative to pGL3. Extraction of ^{125}I -pGL3 from PK time points, followed by agarose gel electrophoresis and autoradiography demonstrates that **PM** polyplexes are stable in the blood over 2 hrs. **SV** and **SA** polyplexes are only stable for 20–30 minutes, and pGL3 is digested within 1 min. Panel B illustrates the relative PK profile for PEG-peptides of differing length. Panel C illustrates the influence of C-terminal (**SM1**_{5kDa}), N-terminal (**SM3**_{5kDa}) and middle (**SM4**_{5kDa}) PEG attachment in comparison to **S_{alk}** polyplexes that lack PEG. The corresponding calculated PK parameters are included in Table 2S. Each curve represents the mean and standard deviation from dosing triplicate mice serially sampled at the times indicated.

Figure 6. Biodistribution Analysis of PEGylated Polyacridine Peptide Polyplexes

The biodistribution of polyplexes was determined by dosing triplicate mice. Panel A illustrates the percent of dose recovered in the major tissues at a biodistribution time of 5 min. The asterisks indicates statistical significance of $p < 0.05$ compared with **SM1_{5kDa}**. Panels B, C, and D illustrate the percent of dose in the liver, lung, and spleen at biodistribution times ranging from 5 min to 6 hrs. Inset B' illustrates a subset of the results from Panel B. Each bar represents the mean and standard deviation of three determinations.